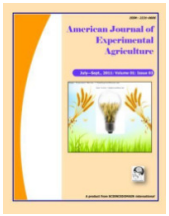

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Exogenous Cytokinin Promotes *Epipremnum aureum* L. Growth through Enhanced Dry Weight Assimilation rather than through Changes in Partitioning

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Benzylaminopurine (BAP) sprays have been shown to increase leaf size and leaf appearance rate, as well as biomass accumulation in pot-grown *Epipremnum aureum* L. BAP-mediated enhanced growth could either be the consequence of a higher investment of dry weight in leaf area development thus leading to a positive dry weight accumulation feedback, to a promoting effect on dry weight assimilation per unit leaf area.
Study Design: A randomized complete block factorial design with three blocks was used.

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Place and Duration of Study: Two experiments were carried out in a greenhouse at the Faculty of Agronomy, University of Buenos Aires, Argentina (34°28'S) from the 8th September 2007 and 5th September 2008, respectively, to the 12th March 2008 and 11th March 2009 respectively.
Methodology: We analyzed the effect of exogenous BAP supplied in different number of applications and at different concentrations under three light intensities, on dry weight accumulation and partitioning in *E. aureum* grown in pots, in two greenhouse experiments.
Results: A single 5mg L^{-1} BAP application was enough to increase the dry weight accumulation rate in comparison to untreated controls, irrespective of the light intensity. A strong direct relationship between the relative growth rate (RGR) and the net assimilation rate (NAR) were found, while an inverse relationship was observed between RGR and the leaf area ratio (LAR). Even though BAP increased dry weight partitioning to the aerial part, as revealed by shoot *vs.* root allometric analysis, this did not result in a LAR increase, but rather in higher stem dry weight accumulation, in association with a decrease in the leaf area partitioning coefficient (LAP). NAR promotion by BAP was associated with an increased N content per unit leaf area, rather than with changes in chlorophyll content.
Conclusion: Our results on the ornamental shade plant *E. aureum* also provide information which may help to increase productivity to this crop from a grower perspective.

Keywords: Cytokinin; foliage plant; leaf area partitioning; relative growth rate.

1. INTRODUCTION

Ornamental shade plants usually show low relative growth rates in terms of fresh weight, dry weight and leaf area [1]. The fact that this type of plants are usually grown in pots further limits their growth due to the root restriction syndrome, which has been associated with a reduced supply of cytokinins by the root [2]. In a previous paper [3], we have shown that exogenous benzylaminopurine (BAP) supply to pot-grown rooted-cuttings of *Epipremnum aureum* resulted in a promotion of shoot development, which included increases in final leaf area, in the rate of leaf appearance and in fresh weight accumulation. Since the dry matter content in tissues was unaffected by exogenous hormone supply, the increase rate in fresh weight accumulation indicates an increase in dry weight-based to the relative growth rate (RGR). In general, variation in RGR may be explained by changes in either the net assimilation rate (NAR) or in the leaf area ratio (LAR), depending on the source of variation in RGR. For example, when the cause of RGR variation is light intensity, NAR has been shown directly correlated with RGR [4] while an inverse relationship between LAR and RGR can be found [5]. On the other hand, working with 24 wild species, Poorter et al. [6] reported that differences in LAR explained species-inherent variation in RGR under comparable growing conditions, although Shipley [7] proposed that the effect of LAR and NAR could vary depending on light intensity (NAR becoming more important at higher irradiances). Furthermore, work on *Aegilops* and *Triticum* species revealed that the contribution of NAR and LAR to changes in RGR depended on the time-scale of measurements probably associated with the developmental stage of the crop [8]. Regarding the effect of exogenous BAP, available evidence does not allow to discern whether changes in LAR or NAR can explain a promoting effect of the hormone on RGR.

Cytokinin-driven diversion of assimilates and mineral nutrients towards shoot meristems, rather than to roots, ultimately resulting in a differential increase in aerial biomass has been reported for a wide number of species [9-13], including ornamental plants [14,15]. Plant tissues and organs rich in cytokinins, like the stem apical meristem, are known to attract photoassimilates [16]. Whether the promoting effect of cytokinins on shoot growth can be explained by an increased partitioning of resources to the aerial part should be tested by calculating the allometric coefficient between shoot and roots. Furthermore, cytokinins also affect meristem function and stem-cell identity in the center of shoot meristems [17] and exogenous supply of BAP to *E. aureum* plants has been shown to increase final leaf size and accelerate the rate of leaf appearance [3]. The promotion of leaf area development is an especially important "reinvestment" because it drives growth in an exponential fashion. One important parameter that accounts for this effect by relating leaf area development to available assimilates is the leaf area partitioning coefficient (LAP) [18]. Both changes in the shoot *vs.* root allometric coefficient, and/or in LAP, might explain changes

in LAR that would ultimately explain the promoting effect of BAP on dry matter accumulation; however no reports about possible effects of cytokinins on these growth parameters are available in literature.

On the other hand, cytokinins are also known to enhance carbon fixation per unit leaf area [19]. Exogenously applied cytokinin to detached barley leaves has been shown to stimulate transcription of a wide number of chloroplast genes, including rbcL, which codes for Rubisco large subunit [20]. Cytokinins are also involved in chlorophyll biosynthesis [21]. Moreover, exogenous application of cytokinin [22] and overexpression of endogenous cytokinin in transgenic plants [23] have been reported to increase leaf thickness, which may further enhance dry weight accumulation per unit leaf area. Therefore, at least a part of the promoting effects of BAP application on RGR may be mediated by an increase in NAR. Besides, cytokinin effects on photosynthetic efficiency are mostly evident in low light intensity environments [10]. The cytokinin effect on chloroplast transcription depends on the presence of light [20]. In agreement with this, the exogenous supply of cytokinin in field environments is not always effective in promoting dry weight fixation; i.e. Hosseini et al. [24] found no increase in the photosynthetic rate or chlorophyll content of *Hordeum vulgare* of BAP-sprayed plants except at the late period of grain filling. In greenhouse grown *E. aureum*, BAP was found to promote leaf growth under three different light intensities, but the effect was especially high under the intermediate one [3]. Therefore, the magnitude of a possible promotion of NAR by BAP may depend on light intensity.

In this work we analyzed whether changes in dry weight assimilation and/or partitioning may explain the BAP-induced growth promotion of *E. aureum* rooted cuttings grown under greenhouse conditions with different light intensities and a number of BAP applications.

2. MATERIALS AND METHODS

2.1 Plant Material, Treatments and Experiments

Rooted cuttings of *E. aureum* were transplanted into 1.2L plastic pots (one cutting per pot) filled with a 1:1 (v/v) mix of *Sphagnum maguellanicum* peat and river waste [25]. Plants were watered daily and fertilized weekly with N, P, K and Ca fertilizer added to the irrigation water (50mg L^{-1} N) (1.0:0.5:1.0:0.5 N:P:K:Ca). Two experiments were carried out in a greenhouse at the Faculty of Agronomy, University of Buenos Aires, Argentina (34°28'S) from the 8th September 2007 and 5th September 2008, respectively, to the 12th March 2008 and 11th March 2009 respectively. The greenhouse was covered with a neutral black shade-cloth (for 50% full sunlight) (Agriplast S.A. Buenos Aires, Argentina) for the experiment in 2007-2008. In the 2008-2009 experiment, the greenhouse was divided into nine compartments with different neutral black shading clothes (Agriplast S.A. Buenos Aires, Argentina) to obtain irradiances equivalent to 70%, 50%, or 30% of full sunlight (three compartments per light intensity). Light quality was not changed significantly (data not shown) by the shade-clothes, as confirmed using a 660/730 sensor (Skye instruments, Wales, UK). Daily mean temperatures (°C) and light intensities (mol photons m^{-2} day^{-1}) for the different experiments were recorded with three HOBO sensors (Onset Computer Corp. Bourne, MA, USA) connected to HOBO H8 data loggers. The plants arrangement at a density of 25 plants m^{-2} avoided mutual shading.

The benzylaminopurine (BAP) (SIGMA EC 214-927-5) water solutions were applied by spraying all leaves to run-off at sunset (no wetting agent was added). For the 2007-2008 experiment plants were sprayed with 0 (control) or 5mg L^{-1} BAP solutions 7 days after transplanting and spray was repeated one and two months afterwards, rendering four different treatments, 0-0-0; 5-0-0, 5-5-0 and 5-5-5mg L^{-1} BAP, which correspond to 0, I, II and III BAP applications, respectively. BAP concentration was chosen from a previous experiment performed in 2006-2007 [3]. In the 2008-2009 experiment plants were sprayed with 0 (control), 2.5, 5, 10, or 50 mg L^{-1} BAP solutions 7 days after transplanting.

2.2 Growth Evaluations

For destructive measurements, six plants per treatment and sampling date were randomly chosen at the beginning of the experiments (transplant stage) and 60, 90, 120, 150 and 180 days after transplanting. Separate plant parts (two plants per block) were dried at 80°C for 48 hours and weighed to obtain the dry weight of shoots (including leaf blades, petioles and stems) and roots. Leaf area was determined with a LI-COR 3000A (LI-COR Inc., Lincoln, NE, USA) automatic leaf area meter, Leaf area at zero time

and the relative leaf area expansion rate (RLAER) were calculated from the regression of the natural logarithm of total leaf area *vs*. time (in days).

Whole plant RGR was calculated as the slope of the linear regression of the natural logarithm of dry weight *vs.* time (in days). The net assimilation rate (NAR) which represents the rate of dry weight increase per unit leaf area and per unit time, and the leaf area partitioning coefficient (LAP), which represents the change in the leaf area ratio per unit time and, thus estimates the partitioning of assimilates into new leaf, were calculated according to Potter and Jones [18] as following:

$$NAR = (k_w W_0 e^{k_w t}) / (A_0 e^{k_a t})$$

$$LAP = (k_a A_0 e^{k_a t}) / (k_w W_0 e^{k_w t})$$

where: k_w: RGR (days^{-1}); W_0: extrapolated value of total dry weight at time zero (g); A_0: extrapolated value of leaf area at time zero (cm^2); k_a: RLAER (days^{-1}); t: time (in days) at the midpoint of the experimental period and e: base of natural logarithms.

Mean LAR was calculated as RGR/NAR. The components of LAR, specific leaf area (SLA) and leaf weight ratio (LWR) were obtained as follows: SLA was calculated as the ratio of individual leaf area to leaf dry weight (average of all plant leaves) while LWR was calculated as the ratio of whole plant leaf dry weight to whole plant dry weight, in both cases at the final sampling of the 2008-2009 experiment.

The allometric coefficients between root and shoot and between leaf blades and the petiole-stem fraction were calculated as the slope (β) of the straight-line regression of ln root dry weight vs. ln shoot dry weight (ln Root dry weight = $\alpha + \beta$ x ln Shoot dry weight) and between ln leaf blade dry weight and ln (petiole+stem) dry weight (ln Leaf blade dry weight = $\alpha + \beta$ x ln Petiole-Stem dry weight), respectively. For root-shoot allometry, data from the first sampling were excluded of the analysis because of their departure from linearity, which was likely the consequence of transplant effects on the root: Shoot ratio.

Chlorophyll analysis was performed on the youngest fully expanded leaf from plants at the final sampling of both experiments. Leaf disks were cut from the central area near the mid-vein of each leaf and placed in vials containing 3cm^3 of N, N-dimethylformamide. Leaf disks were vacuum-infiltrated and stored for three days in complete darkness. At this time, chlorophyll had completely eluted to the solvent and absorbance was measured at 647nm and 664nm using a Metrolab 1600 spectrophotometer. Chlorophyll content was calculated as indicated by Inskeep and Bloom [26].

Nitrogen analysis (Kjeldall method) was performed on the youngest fully expanded leaf from plants at the final sampling of the 2008-2009 experiment with a LB-UDK129 analyzer (Labometric, Buenos Aires, Argentina). Nitrogen was determined only in plants treated with 0 or 5mg L^{-1} BAP.

2.3. Statistical Analysis

For growth analysis in both 2007-2008 and 2008-2009 experiments, plants were arranged in a randomized complete block design with three blocks and two plants per block, for each treatment and sampling date. Data were subjected to one-way ANOVA (2007-2008 experiment) or two-way ANOVA (2008-2009) experiment. For chlorophyll and nitrogen analysis, a similar experimental design was used, except that only one plant per block was sampled. Means were separated by Tukey's test ($P<0.05$). For allometric analysis, differences in slopes were tested using the SMATR package [27], since no significant block effects were found in any case, data points from all individual plants were included in this analysis.

3. RESULTS

3.1 Climate

The air temperatures ranged between 10.28-20.15°C (minimum) and 20.94-30.08°C (maximum) during the 2006-2007 experiment, between 12.68-20.69°C (minimum) and 21.38-30.62°C (maximum) during the 2007-2008 experiment and between 10.48-20.60°C (minimum) and 18.63-31.13°C (maximum) during the 2008-2009 experiment. Solar radiation ranged between 7.61 and 10.71MJ m^{-2} day^{-1} during the 2006-2007 experiment, between 6.34 and 10.99MJ m^{-2} day^{-1} during the 2007-2008 experiment and between 5.93 and 11.79MJ m^{-2} day^{-1} during the 2008-2009 experiment (Table 1).

3.2 BAP Applications Number

An increased accumulation of dry weight was observed in plants sprayed once, twice or three times with 5mg L^{-1} BAP, in comparison to controls and the response was highest when BAP was applied just once. The effect of BAP was generally higher in shoots than in roots (Fig. 1), leading to decreased root: Shoot ratios from 0.56 in controls to 0.42 in BAP treated plants (Table 2).

Accordingly, the highest RGR values were found with only one BAP application, although all BAP treatments led to higher RGR than control plants. NAR was also highest in plants sprayed just once and decreased with subsequent BAP applications to a value close to control in plants sprayed three times. In fact, NAR was more promoted by BAP than RGR in plants sprayed just once (i.e. 27.3% vs. 18.9% increases in NAR and RGR, respectively) Conversely, plants sprayed just once had lower mean LAR and LAP values than controls and both variables tended to increase with further BAP sprays towards the values exhibited by control plants (Table 2). A close direct relationship between RGR and NAR (r^2=0.972, P<0.001) could be observed by plotting data from all treatments, while an inverse relationship between RGR and LAR [LAR = -3.71 RGR + 153.32 (r^2=0.773, P<0.01)] was observed (Fig. 2).

The allometric analysis between roots and shoots showed that BAP application increased dry weight partitioning to shoots, as revealed by lower values of the coefficient β. Within shoots, BAP sprays tended to increase partitioning towards the stem-petiole fraction (Table 3).

There were no significant changes in chlorophyll content per unit leaf area or dry weight, except for a decrease found with three BAP applications when chlorophyll content was expressed on a dry weight basis (Table 4).

3.3 BAP and Light Integral Relationships

The response to BAP sprays was evaluated under different light intensities. Whole plant dry weight accumulation was promoted by BAP under the three environments assayed (70, 50 and 30% full sunlight), but differences in optimal concentrations and magnitude of the response varied among light treatments and significant interactions (BAP concentration x light intensity) were observed according to ANOVA (P<0.001 for stem and root, and P<0.05 for leaf and petiole dry weight) (data not shown). Under 70% full sunlight, the highest response was achieved with 2.5mg L^{-1} BAP (Fig. 3a). Plants under 50% full sunlight accumulated dry weight at a substantially lower rate than under 70% full sunlight; however, the promotion of dry weight accumulation by BAP, in relation to control plants, was relatively higher than under 70% full sunlight; the highest effect was observed with 5mg L^{-1} BAP (Fig. 3b). Under the lowest irradiance (30% full sunlight), a low dry weight accumulation was observed in control plants and the maximum promotion of dry weight accumulation was achieved with 5mg L^{-1} BAP (Fig. 3c).

Table 1. Monthly patterns of daily minimum and maximum temperatures and mean daily photosynthetically active radiation (PAR) integral during the three experimental seasons

	Minimum temperature (°C)			Maximum temperature (°C)			PAR (mol photons m^{-2} d^{-1})		
	2006-2007	2007-2008	2008-2009	2006-2007	2007-2008	2008-2009	2006-2007	2007-2008	2008-2009
September	10.28	12.68	10.48	20.94	21.38	18.63	7.61	6.34	5.93
October	14.78	14.37	13.27	24.68	23.75	23.26	8.94	8.15	8.78
November	15.61	12.71	19.49	25.48	24.97	30.00	10.17	10.50	10.56
December	19.57	18.09	19.31	30.03	29.30	29.46	10.71	10.99	10.79
January	20.15	20.69	20.60	29.39	30.62	31.13	9.88	10.46	11.16
February	19.72	20.68	19.39	30.08	29.90	29.90	8.36	8.66	9.74
March	18.03	17.64	19.45	25.97	26.56	28.18	5.85	6.82	7.70

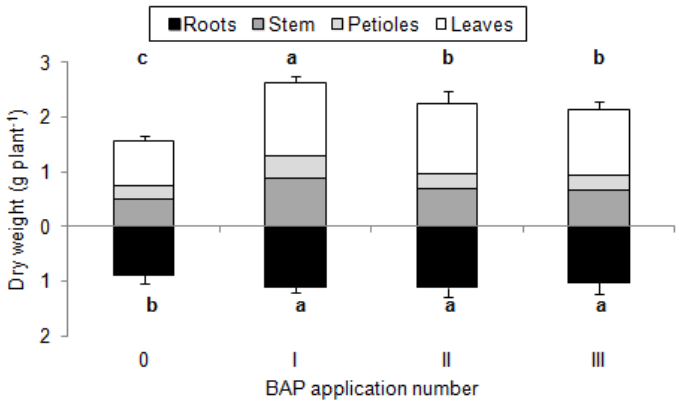


Fig. 1. Mean dry weights (n=3) of different plant parts (roots, stem, petioles, and leaves) at the end of the 2007-2008 experiment in *E. aureum* plants subjected to different number of applications of 5mg L^{-1} BAP. Bars indicate standard errors. Different letters indicate statistical significance (*P*<0.05) for total aerial biomass and roots

Table 2. Effect of a different number of 5mg L^{-1} BAP applications on RGR, NAR, LAR, LAP and root: shoot ratio (2007-2008 experiment). Mean values (n=3) in each column followed by a different lower-case letter indicate significant differences (*P*<0.05). The probability of the slope being zero was P = 0.001 for all growth parameters

BAP applications	RGR (mg g^{-1} day^{-1})	NAR (g cm^{-2} day^{-1}) (x 10^{-5})	LAR (cm^2 g^{-1})	LAP (cm^2 day^{-1}) (g day^{-1})	Root: Shoot ratio
0	12.34^{c}	11.62^{c}	105.86^{a}	121.35^{a}	0.56^{a}
I	14.67^{a}	14.80^{a}	98.68^{b}	104.76^{c}	0.42^{c}
II	13.51^{b}	13.12^{b}	102.88^{a}	113.55^{b}	0.49^{b}
III	12.98^{b}	11.91^{c}	108.29^{a}	123.40^{a}	0.48^{b}

In agreement with this, BAP sprays increased RGR under the three light environments tested, but promotion was relatively higher at the intermediate light intensity. In parallel, NAR generally increased and LAR and LAP decreased with BAP treatment (Table 5). The promotion of NAR by BAP treatment tended to be higher than that of RGR (i.e. 32.9% vs 18.6% increases in NAR and RGR, respectively, by 5mg L^{-1} BAP under 70% full sunlight). RGR of control plants decreased with a decreasing light intensity and this effect was generally accompanied by a decrease in NAR and an increase in both LAR and LAP. Furthermore, SLA and LWR increased with decreasing light intensity, while BAP sprays tended to reduce their values (Table 5).

When data from different BAP treatments and light environments were plotted together; a close direct relationship between RGR and NAR (r^2= 0.944, *P*<0.001, Fig. 4a) was found, while a significant inverse relationship between RGR and LAR (r^2= 0.488, *P*<0.01, Fig. 4b) was observed. Besides, significant direct relationships between SLA and LAR (r^2=0.263, *P*<0.01, Fig. 4c) and between LWR and LAR (r^2=0.428, *P*<0.01, Fig. 4d) were observed.

BAP application determined a general decrease in the allometric coefficient **β** between roots and shoots under the three light environments, although at the highest BAP concentrations under 50% full sun, differences with respect to controls were not significant (Table 6) ANOVA revealed a significant (*P*<0.001) BAP concentration x light intensity interaction (not shown). The influence of BAP sprays on the allometric pattern within the aerial part was generally lower than that on root: Shoot allometry, but nevertheless significant decreases in the allometric coefficient **β** for the leaf blades vs. stems + petioles relationship were found in plants treated with BAP at low concentrations, especially under the lowest light intensities (Table 6). Again, in this case, ANOVA revealed a significant (*P*<0.001) BAP concentration x light intensity interaction (not shown).

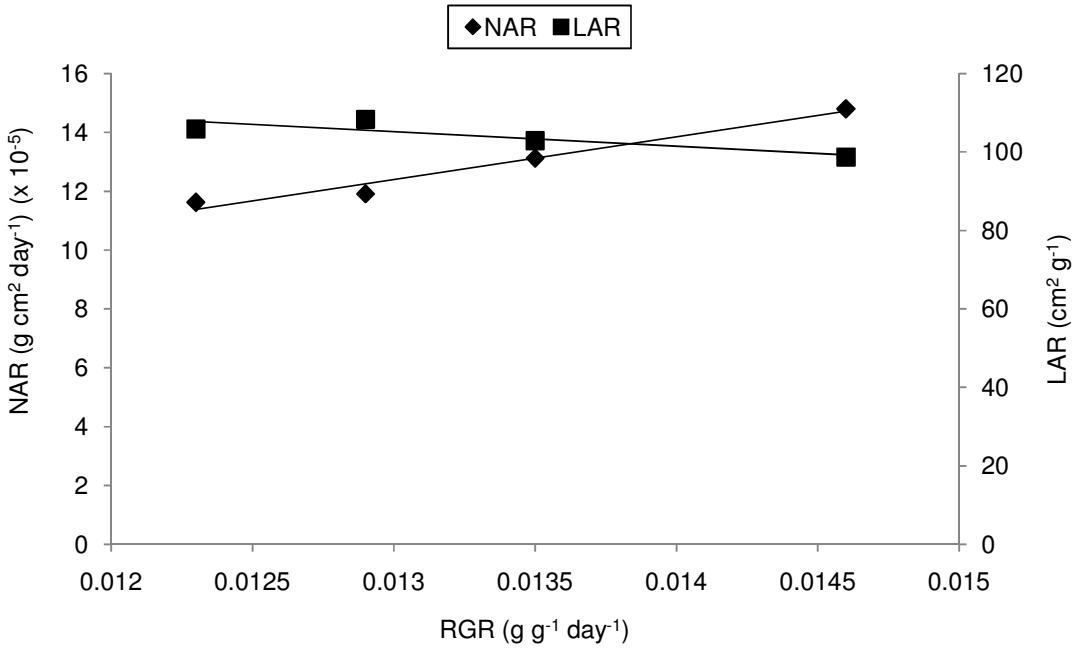


Fig. 2. Changes in the net assimilation rate (NAR) and the leaf area ratio (LAR) related to the relative growth rate (RGR). The straight-line regressions were: NAR x 10^{-5} = 1.45 RGR–6.46 (r^2 = 0.972, P<0.001) and LAR = -3.71 RGR + 153.32 (r^2 = 0.773, P<0.001)

Table 3. Allometric relationships between shoots and roots (ln Root dry weight = α + β x ln Shoot dry weight) (n=30) and between leaf blades and petioles-stems (ln Leaf Blades dry weight = α + β x ln Petioles + Stems dry weight) (n = 36) in *E. aureum* cuttings subjected to a different number of 5mg L^{-1} BAP applications (2007-2008 experiment). Mean values in each column followed by a different lower-case letter indicate significant different slopes according to SMATR analysis. The probability of the slope being zero was *P*<0.001

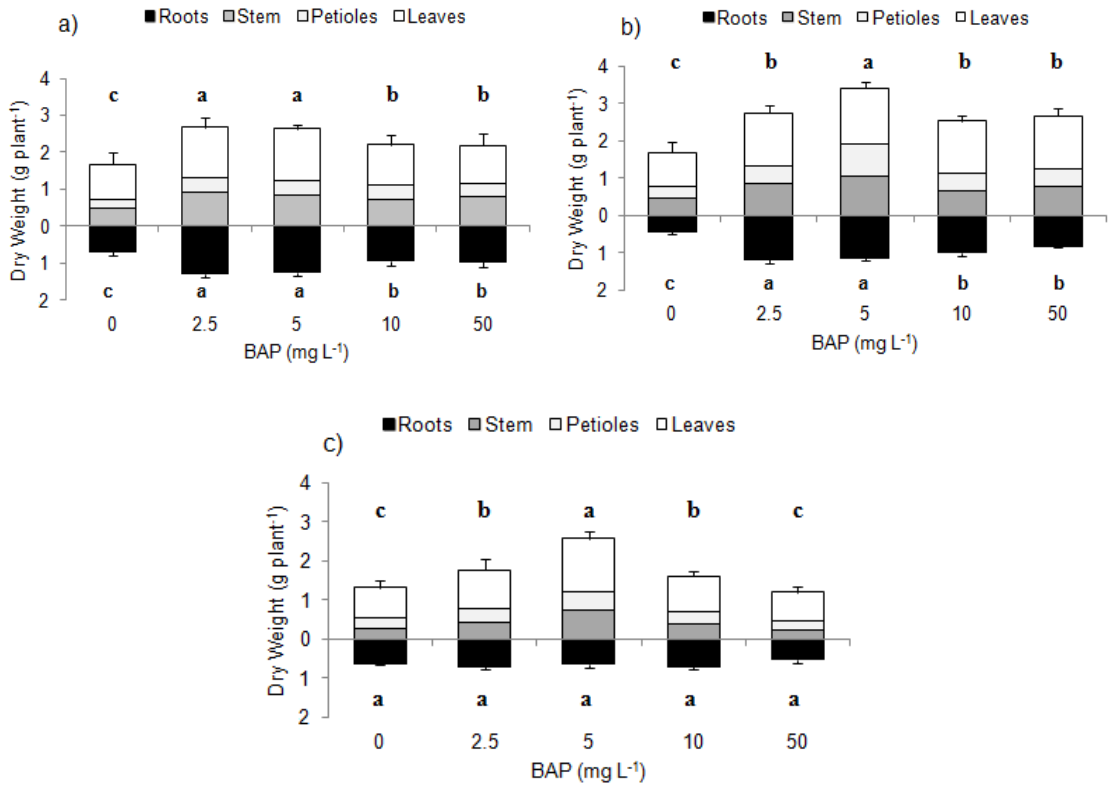
BAP applications	Roots vs. Shoots			Leaf blades vs. Petioles-Stems		
	α	β	r^2	α	β	r^2
0	-0.57	0.79[a]	0.74	0.40	1.17[a]	0.87
I	-0.36	0.54[b]	0.66	0.22	1.11[b]	0.99
II	-0.64	0.57[b]	0.61	0.18	1.10[b]	0.98
III	-0.56	0.48[c]	0.73	0.41	1.17[a]	0.96

Table 4. Effect of a different number of 5mg L^{-1} BAP applications on chlorophyll content of *E. aureum* leaves at final harvest (2007-2008 experiment). Mean values (n=3) in each column followed by a different lower-case letter indicate significant differences at *P*<0.05

BAP applications	Chlorophyll content	
	(μg cm^2)	(mg g^{-1} dry weight)
0	33.5[a]	12.78[a]
I	36.2[a]	12.94[a]
II	38.1[a]	12.99[a]
III	27.0[a]	9.60[b]

Chlorophyll concentration per unit dry weight was not significantly affected by BAP application, except for minor variation observed under the lowest irradiance. However, when expressed per unit leaf area, chlorophyll content generally increased in BAP-treated plants (especially with low BAP concentrations) with respect to controls (Table 6). This effect may be attributed to the BAP-driven moderate decrease in SLA, shown in Table 4. Besides, chlorophyll concentration tended to increase with a decreasing irradiance (Table 7).

Nitrogen concentration (mg N dry weight^{-1}) was slightly (about 7%) but significantly increased by 5mg L^{-1} BAP treatment, under the three irradiances tested. When expressed on a leaf area basis this effect was even more pronounced (20-25%) (Table 8), which is again attributable to a decrease in specific leaf area of BAP-treated plants.



ANOVA

Source of variation	Leaves	Petioles	Stems	Roots
BAP concentration	***	***	***	***
Light intensity	**	**	***	***
BAP concentration x Light Intensity	**	**	***	***

*Significance *** 0.001; ** 0.01*

Fig. 3. Mean dry weight (n=3) from different plant parts (roots, stem, petioles and leaves) at the end of the 2008-2009 experiment in *E. aureum* plants grown under three light environments: 70% (a), 50% (b) and 30% (c) full sunlight and sprayed with different BAP concentrations. The standard errors over each bar and the significance of interactions (ANOVA) have been indicated. Different letters indicate statistical significance (*P*<0.05) based on total aerial biomass and roots

4. DISCUSSION

Ornamental plants like *E. aureum* are commonly grown in pots for commercial purposes. Under these conditions, roots are impeded to develop normally [28] and this restriction would be associated with a limited production of cytokinins [29] which in turn negatively affects the development of the aerial part [11]. In a previous paper [3] we showed that this effect can be reverted by exogenous cytokinin supply and that a single spray of 5mg L^{-1} BAP on *E. aureum* potted plants promoted leaf area and fresh weight accumulation. In the present work we show that BAP also increased RGR, which in turn depended on a number of applications (Table 2), BAP concentration and light environment (Table 5).

To investigate whether the BAP-driven increase in RGR could be explained either by an increased resource investment in the development of leaf area or by an increased efficiency of dry weight fixation per unit leaf area, a classic growth analysis approach was used. Apriori, both alternatives appeared as likely since there is abundant experimental or bibliographic support: In our previous paper [3] it was shown that BAP increased *E. aureum* final leaf size and decreased the phyllochron, but cytokinins are also well known to promote the efficiency of the photosynthetic apparatus [30].

RGR is the product of LAR, the so-called 'morphological component' and NAR, the 'physiological component'. A change in dry weight partitioning towards the development of leaf area would be reflected in an increased LAR, while an increased efficiency of dry weight fixation would be associated with higher NAR values, since this variable is largely the net result of dry weight gain and dry weight losses [31]. In our experiments one single application of 5mg L^{-1} BAP led to an average increase in RGR of 19.4% and 18.7% in the 2007-2008 and 2008-2009 experiments respectively and this effect was associated with an increase in NAR of 35.6% and 27.3% with a relatively slight decrease in LAR of 12.0% and 13.7% respectively (Tables 2 and 5-70% full sunlight). This negative effect of BAP spray on LAR was somewhat unexpected, since treated plants developed larger leaves and also more leaves per unit time [3] and also promoted lower root: Shoot ratios. Moreover, a close direct relationship between RGR and NAR (Figs. 3 and 4a) could be observed plotting data from all treatments of Tables 2 and 5 while an inverse relationship was found between RGR and LAR (Figs. 3 and 4b). Thus, BAP sprays enhanced NAR even more than RGR. On the other hand, the BAP-induced decrease in LAR could be attributed mainly to lower LWR (which measures the allocation of biomass to leaves *vs.* other plant parts) [31] in treated plants, but also to changes in SLA (Fig. 4d).

The strong BAP-mediated increase in NAR could be the consequence of different possible effects of cytokinin on the efficiency of carbon fixation. Cytokinins have long been implicated in chlorophyll synthesis [30] although this not always results in an increased concentration in plant tissues [32]. In our experiments, chlorophyll concentration per unit dry weight was unaffected by BAP treatment. Cytokinins also appear to increase nitrogen content in plants [33,34] and in accordance with this, an increased leaf nitrogen concentration per unit dry weight was found as a consequence of 5mg L^{-1} BAP treatment (Table 8). Although we have not attempted to determine whether or not part of the increased nitrogen concentration corresponds to enzymes involved in carbon fixation, this possibility seems likely, since cytokinins have been reported to promote synthesis of Rubisco [35]. Furthermore, BAP might promote leaf blade thickening, thus leading to an increase in the levels of photosynthetic machinery per unit leaf area [36]. SLA reflects biomass per unit leaf area and is well correlated to leaf thickness [37]. Changes in SLA as a consequence of BAP treatment were relatively small, however a decreasing trend was observed, which reflects a moderate leaf thickening (Table 5) and this contributed to a slight increase in chlorophyll and especially in nitrogen content per unit leaf area. Therefore, it seems likely that NAR promotion by BAP is related to an increased efficiency of carbon fixation per unit leaf area, similarly as reported for tobacco by Werner et al. [38].

To further explain the observed decrease in LAR in BAP-treated plants, the relationship between leaf development and dry weight partitioning was analyzed. First, values of the LAP coefficient, a parameter which was originally proposed to evaluate dry weight investment on the development of leaf area [18] decreased as a consequence of BAP treatment (Tables 2 and 5).

Table 5. Effect of a single BAP spray at different concentrations on RGR, NAR, LAR, LAP, SLA, LWR and root: shoot ratio of *E. aureum* plants grown under different light intensities (70%, 50% and 30% full sunlight) (2008-2009 experiment). Mean values (n=3). Different lower-case letters indicate significant differences (*P*<0.05) between BAP concentrations while different capital letters indicate significant differences (P = 0.05) for each BAP concentration between different light intensities. The probability of the slope being zero was P = 0.001 for all growth parameters

Light Intensity	BAP (mg L^{-1})	RGR (mg g^{-1} day^{-1})	NAR (g cm^{-2} day^{-1}) (x 10^{-5})	LAR (cm^2 g^{-1})	LAP (cm^2 day^{-1}) / (g day^{-1})	SLA (cm^2 g^{-1})	LWR (g g^{-1})	Root: Shoot ratio
70% full sun	0	12.35^{cA}	10.96^{cA}	112.27^{aA}	131.44^{aC}	336.37^{aB}	0.37^{bC}	0.43^{aB}
	2.5	14.61^{aA}	13.59^{aA}	104.49^{bB}	114.79^{bB}	333.31^{aB}	0.36^{bB}	0.41^{aA}
	5	14.65^{aA}	14.57^{aA}	100.23^{bC}	107.79^{bC}	299.83^{bB}	0.34^{bC}	0.41^{aA}
	10	13.10^{bA}	12.47^{bA}	105.10^{bB}	117.13^{bC}	290.19^{bB}	0.43^{aA}	0.43^{aA}
	50	13.32^{bA}	11.98^{bA}	111.06^{aB}	127.76^{aC}	274.89^{bB}	0.43^{aA}	0.40^{aA}
50% full sun	0	9.36^{cB}	7.78^{cB}	119.49^{aA}	154.18^{aB}	371.97^{aA}	0.48^{aA}	0.43^{aB}
	2.5	13.50^{aB}	12.69^{aB}	106.36^{bB}	115.81^{cB}	327.38^{bB}	0.39^{bB}	0.35^{bB}
	5	14.65^{aA}	12.74^{aB}	114.57^{aB}	132.62^{bB}	318.32^{bA}	0.47^{bA}	0.33^{bB}
	10	12.28^{bB}	11.02^{bB}	110.75^{aB}	128.00^{bB}	316.09^{bA}	0.39^{cB}	0.34^{bB}
	50	$14,50^{aA}$	12.53^{aA}	115.71^{aB}	134.07^{bB}	338.18^{bA}	0.43^{bA}	0.31^{bB}
30% full sun	0	7.82^{cC}	6.38^{dC}	122.17^{aA}	170.72^{aA}	373.53^{aA}	0.46^{aB}	0.49^{aA}
	2.5	11.79^{aC}	9.97^{aC}	118.34^{aA}	145.42^{bA}	357.98^{aA}	0.43^{aA}	0.40^{bA}
	5	11.86^{aB}	9.18^{bC}	124.14^{aA}	158.98^{bA}	329.11^{bA}	0.40^{bB}	0.40^{bA}
	10	10.88^{aC}	8.92^{bC}	114.38^{aA}	144.65^{bA}	315.39^{bA}	0.41^{bB}	0.38^{bB}
	50	10.11^{bB}	8.29^{cB}	120.59^{aA}	156.77^{bA}	343.76^{bA}	0.43^{bA}	0.41^{bA}

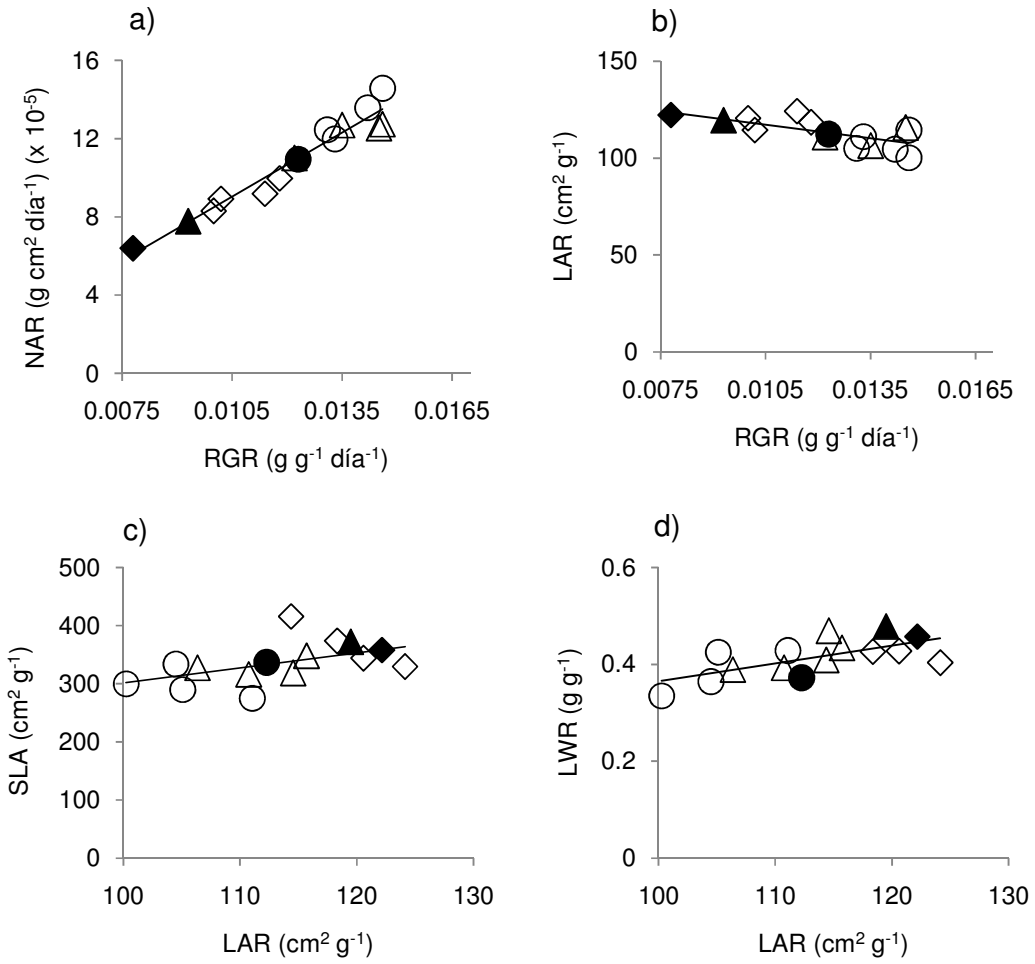


Fig. 4. Relationships between NAR (gcm^{-2} day^{-1} x 10^{-5}) (A), LAR (cm^2 g^{-1}) (B) and RGR (mg g^{-1} day^{-1}) and between SLA (cm^2 g^{-1}) (C) or LWR (g g^{-1}) (D) and LAR (n = 3). Linear regression equations are: NAR x 10^{-5} = 1.092 RGR – 2.43 (r^2 = 0.944, P<0.001); LAR = - 2.34 RGR + 141.88 (r^2 = 0.488, P<0.01); SLA = 2.58 LAR + 43.53 (r^2 = 0.363, P<0.01); LWR = 0.0037 LAR + 0.0003 (r^2 = 0.428, P<0.01). ●, ○: 70%; ▲, ∆: 50% and ♦, ◊: 30% full sun. Controls: close symbols; BAP-sprayed: open symbols

Second, an analysis of the allometric ratios between roots and shoots and between leaves and stems + petioles was carried out. We have analyzed stems and petioles together because both tissues derive from the same group of cell at the apex [39]. Changes in the allometric ratios reflect variation in dry weight partitioning [40], and partitioning of resources between competing sites is fundamental to plant growth; especially because plants are capable of modifying their resource allocation to favor the development of their growing parts [41]. This analysis showed that BAP applications, at least at the concentrations that are most effective in promoting growth, led to an increase in partitioning towards the aerial part but that, within the latter, BAP increased the partitioning towards stem and petioles rather than to leaf blades (Tables 3 and 6). This is presumably associated with thicker stems in BAP-sprayed plants and in turn it could be related to the promoting effect of BAP on the rate of leaf appearance in *E. aureum*, which was reported in a previous paper [3]. It has been suggested that cytokinins control growth mainly through the regulation of cell division and differentiation in the stem apical meristem [42] and that the size of the apical meristem is in turn related to the rate of leaf appearance, as shown in experiments with cytokinin deficient *Arabidopsis* mutants [16].

Table 6. Allometric relationships between roots and shoots (ln Root dry weight = α + β x ln Shoot dry weight) (n = 30) and between leaf blades and petioles-stems (ln Leaf Blades dry weight = α + β x ln Petioles + Stems dry weight) (n = 36) in *E. aureum* plants grown under 70%, 50% or 30% full sunlight and sprayed with BAP at different concentrations (2008-2009 experiment). Mean values in each column followed by a different lower-case letter indicate significantly different slopes between BAP concentrations while different capital letters indicate significant differences (*P*<0.05) for each BAP concentration between different light intensities according to SMATR analysis

Light Intensity	BAP (mg L^{-1})	Roots vs. Shoots			Leaf blades vs. Petioles-Stems		
		α	β	r^2	α	β	r^2
70% full sun	0	-0.57	0.79^{aC}	0.74	0.17	1.00^{bC}	0.98
	2.5	-0.46	0.64^{bB}	0.65	0.06	0.95^{cC}	0.99
	5	-0.44	0.50^{cB}	0.69	0.07	1.02^{bA}	0.95
	10	-0.57	0.58^{bC}	0.60	0.12	1.04^{aA}	0.99
	50	-0.66	0.59^{bB}	0.64	0.16	1.04^{aB}	0.99
50% full sun	0	-1.08	0.80^{aB}	0.64	0.36	1.12^{aB}	0.95
	2.5	-0.64	0.61^{bB}	0.72	0.13	1.04^{bB}	0.99
	5	-1.12	0.62^{bA}	0.73	0.08	0.94^{cA}	0.98
	10	-0.72	0.76^{aA}	0.61	0.17	1.07^{bA}	0.99
	50	-0.90	0.77^{aA}	0.77	0.09	0.98^{bB}	0.99
30% full sun	0	-0.94	0.95^{aA}	0.67	0.71	1.38^{aA}	0.96
	2.5	-0.97	0.80^{bA}	0.77	0.28	1.15^{cA}	0.99
	5	-0.89	0.57^{cA}	0.61	0.13	1.01^{dA}	0.98
	10	-0.93	0.67^{cB}	0.64	0.16	1.08^{dA}	0.99
	50	-0.85	0.49^{dC}	0.63	0.45	1.22^{bA}	0.97

Table 7. Effect of a single BAP spray at different concentrations on chlorophyll content in *E. aureum* leaves at final harvest in plants grown under different light intensities (70%, 50% and 30% full sunlight) (2008-2009 experiment). Mean values (n = 3) followed by different lower-case letters indicate significant differences between BAP concentrations while different capital letters indicate significantly differences for each BAP concentration between different light intensities (*P*<0.05)

Light Intensity	BAP	Chlorophyll content	
	(mg L^{-1})	(μg cm^{-2})	(mg g^{-1} dry weight)
70% full sun	0	33.5^{bA}	11.28^{aB}
	2.5	37.8^{aA}	12.60^{aB}
	5	36.2^{aA}	10.86^{aB}
	10	35.7^{aA}	10.37^{aB}
	50	37.1^{aA}	10.21^{aB}
50% full sun	0	33.2^{bA}	12.36^{aB}
	2.5	37.2^{aA}	12.19^{aB}
	5	37.4^{aA}	11.90^{aB}
	10	33.1^{bA}	10.47^{aB}
	50	37.2^{aA}	12.94^{aA}
30% full sun	0	36.7^{bA}	13.14^{bA}
	2.5	39.6^{aA}	14.80^{aA}
	5	38.9^{aA}	12.81^{bA}
	10	37.3^{bA}	15.48^{aA}
	50	39.0^{aA}	13.42^{bA}

Table 8. Effect of a single 5mg L^{-1} BAP spray on nitrogen content at the final sampling in young, fully expanded leaves of *E. aureum* grown under 70%, 50% or 30% full sunlight (2008-2009 experiment). Mean values (n = 3) followed by different lower-case letters indicate significant differences between BAP concentrations while different capital letters indicate significant differences for each BAP concentration between different light intensities (*P*<0.05)

Light Intensity	BAP	Nitrogen content	
	(mg L^{-1})	(mg N cm^{-2})	(mg N g^{-1} dry weight)
70% full sun	0	0.0654^{bA}	21.8^{bA}
	5	0.0784^{aA}	23.3^{aA}
50% full sun	0	0.0587^{bB}	20.7^{bB}
	5	0.0684^{aB}	22.5^{aB}
30% full sun	0	0.0538^{bC}	19.7^{bC}
	5	0.0675^{aC}	21.7^{aC}

BAP was generally most effective at a low number of applications (2007-2008 experiment) and at low concentrations (2008-2009 experiment). It is well known that plant hormones may promote a given physiological process at low concentrations but the response eventually reaches a plateau and even an inhibition may be found at higher doses. In our experiments, the fact that higher BAP concentrations or a number of applications resulted in a lower growth promotion might be the consequence of a limitation in resources availability to sustain an increased partitioning towards shoots, which was shown to be a part of the BAP response.

As expected, RGR and NAR generally decreased with shading in either control or BAP-treated plants. On the other hand, LAR tended to increase with a decreasing light intensity (Table 5); the latter is a common response of plants growing in poorly illuminated environments, which may be regarded as an adaptive response aimed at maximizing light capture [43]. Applying BAP increased RGR irrespective of the light environment (Table 5) as a consequence of a strong increase in NAR while LAR tended to decrease. In fact, BAP effects on NAR and LAR resembled those observed with an increased illumination in untreated plants, thus suggesting that both light and BAP may act additively, by affecting the same physiological pathway [43]. Thus, when NAR and LAR data from the 2008-2009 experiment were plotted as a function of RGR (Figs. 3, 4a and 4b), all data points were aligned within single linear functions for either NAR or LAR; this is, irrespective of light environment and BAP concentration. The possible additive effect of cytokinins and light are in agreement with the fact that low light intensities decrease root branching [44] and that root apices are the main source of cytokinins to the aerial part [45]. This is also in agreement with the fact that both light and BAP seemed to act additively on leaf nitrogen content (Table 8).

5. CONCLUSION

The results of the present work provide evidence about the mechanisms involved in the growth-promoting role of an exogenously applied cytokinin in *E. aureum*. In general, BAP applications to the aerial part of this species mimicked the reported effects of cytokinins produced naturally by the roots, including higher rates of leaf area production and dry weight accumulation, especially when supplied in low doses (one single application at a low concentration) and under non severe shading. Besides promoting higher RGR, BAP increased dry weight partitioning to shoots, but despite this, the leaf area ratio tended to decrease in treated plants. This could be partly explained because within shoots, dry matter was preferentially allocated to stems rather than to leaves. This was further confirmed by the BAP-induced decrease in the leaf area partitioning coefficient. A strong promotion of the net assimilation rate by BAP was observed, that resulted in RGR promotion despite the decrease in LAR and that could be associated with an increased N content per unit leaf area. Further research on other species is needed to evaluate whether these are general responses of plants to cytokinins and to analyze the physiological mechanisms underlying direct effects of cytokinins on dry weight assimilation. Finally, our results on the ornamental shade plant *E. aureum* also provide information which may help to increase productivity of this crop from a grower perspective.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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